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The Effects of an Inhibiting Factor (Interferon) on the *in vitro* Growth of Granulocyte-Macrophage Colonies

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Summary. Incorporation of preparations of mouse interferon in stimulated cultures of normal mouse bone marrow results in a decrease in the number of colonies developing, and in the appearance of unusual cells in colonies. These unusual cells have the granular, vacuolated cytoplasm of the macrophage, but lobed nuclei more typical of cells of the granulocytic series. The evidence suggests that interferon inhibits the normal multiplication and differentiation of mouse granulocyte-macrophage precursors *in vitro*.

INTRODUCTION

When suitably stimulated, the precursor cells of granulocytes and macrophages from haemopoietic tissues can give rise to colonies of these cell types in semi-solid agar cultures. Stimulation is achieved by the constant presence of a colony-stimulating factor (CSF) in the cultures and sources of this factor for mouse cells are mouse serum, human urine and medium conditioned by mouse embryo cells (Bradley, Metcalf, Sumner and Stanley, 1969; Metcalf, 1970.) CSF from human urine is a glycoprotein of approximately 45,000 molecular weight which retains activity over a wide pH range (Stanley and Metcalf, 1969.) Recent experiments in this laboratory have shown that the injection of mice (McNeill and Killen, 1971) and rabbits (McNeill, Fleming and McCance, 1972) with the synthetic double-stranded polynucleotide Poly I–Poly C can result, not only in an increase in the serum levels of colony-stimulating factor, but also in the appearance of a colony-inhibiting factor (CIF).

Poly I–Poly C is a known inducer of interferon production *in vivo* and *in vitro*. Interferon and colony-inhibiting activity appear simultaneously in sera from mice injected with Poly I–Poly C (McNeill and Killen, 1971) and the interferon and colony-inhibiting activity of different sera are directly proportional (McNeill and Fleming, 1971). Further, interferon and CIF have the same properties with regard to molecular size, and sensitivity to heat, pH and trypsin (McNeill and Fleming, 1971). This communication reports the results of a more detailed investigation into the effects of CIF on colony formation *in vitro*.

MATERIALS AND METHODS

Animals

$C_{57}B1$ mice of both sexes from an inbred colony maintained in this department were used throughout as a source of bone marrow cells for culture, and for preparation of

serum pools after injection of Poly I–Poly C.

Poly I–Poly C

The double-stranded polynucleotide was obtained as a desalted lyophilized product from P-L Biochemicals, Inc., Wisconsin. This material was reconstituted in sterile saline at a concentration of 1 mg per ml and stored at 4°. To ensure adequate complexing, the material was incubated at 37° for 2 hours before animal inoculation.

Preparation of serum interferon

Female $C_{57}Bl$ mice were inoculated intraperitoneally with 100 μg Poly I–Poly C and bled out under ether anaesthesia 3 hours later. The blood was pooled, the serum separated and stored at −20°. Serum pools prepared in this way were labelled PIC-3.

Preparation of CIF

Previous experiments (McNeill and Fleming, 1971) showed that CIF and interferon associated with the albumin peak on Sephadex G-200 chromatography. PIC-3 serum was fractionated in 1-ml aliquots on a Sephadex G-200 column of dimensions 100 cm × 1 cm. Three-ml fractions were collected and the optical density read at 280 nm using an Optika spectrophotometer. Appropriate fractions were combined to form a CIF pool, which was sterilized by filtration through a Millipore filter (pore diameter 220 nm) and stored at −20°.

Bone marrow culture

The methods for the preparation of marrow cell suspensions and the colony culture method have been described (McNeill, 1971). Marrow cells were suspended in 0·3 per cent Eagle's-agar at a concentration of 5×10^4 per ml, and 1 ml of this suspension plated in 30-mm plastic Petri dishes (Nunclon) on a 2-ml base layer of 1·2 per cent Eagle's-agar with colony-stimulating factor (mouse embryo conditioned medium) incorporated. Cultures were incubated at 37° in a sealed humidified box containing 10 per cent CO_2 in air. After 7 days incubation, colonies were counted at a magnification of × 16 using an Olympus dissecting microscope.

Cytological studies were carried out by picking off colonies on to clean microscope slides using a fine Pasteur pipette. Colonies were stained either with 0·6 per cent orcein in 60 per cent acetic acid or May-Grunwald-Giemsa before microscopic examination.

Cell counts on individual colonies were carried out on unstained colonies *in situ* at a magnification of × 40 using an Olympus dissecting microscope.

L-cells

Monolayer cultures of the mouse-derived L-cell (L-929, Flow Laboratories) were prepared in pyrex bottles. Four colony culture cells were suspended in collecting medium after trypsinization and cultured at 200 cells per ml in the same medium as used for bone marrow culture, but without the addition of colony-stimulating factor.

RESULTS

THE EFFECT OF CIF ON COLONY NUMBER

(a) *Dose-response*

The effect of incorporating CIF in cultures on the number of colonies present after

7 days incubation is shown in Fig. 1. These results show that the addition of varying amounts of CIF (0·1 ml serial doubling dilutions per 1 ml agar-cell suspension) to cultures containing a constant amount of stimulating factor (0·06 per cent in underlayer) resulted in a significant decrease in the number of colonies, and that this decrease was dose-dependent. The magnitude of inhibition varied from experiment to experiment, and with different CIF pools, but the dose-response relationship was always apparent.

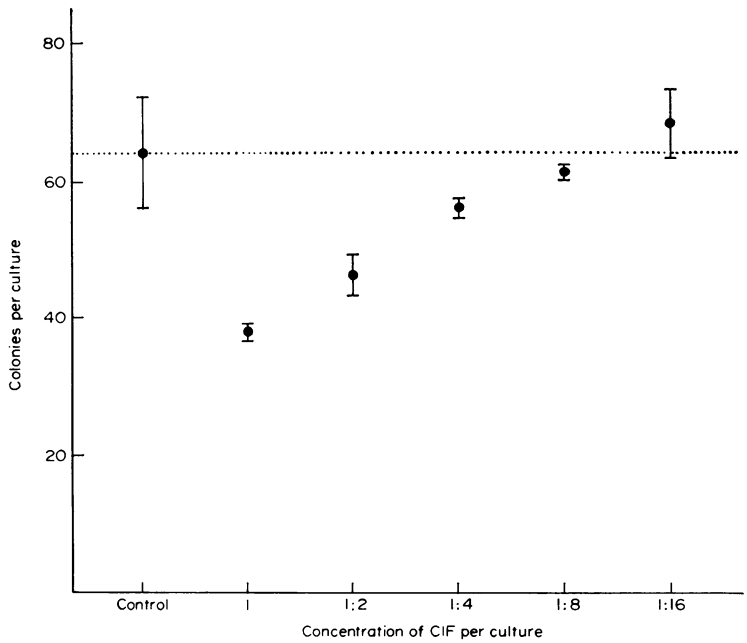

FIG. 1. The effect of incorporating doubling dilutions of colony-inhibiting factor in cultures containing a constant amount of stimulating factor on colony counts after 7 days incubation. (●) Mean of five cultures ±1 SD.

Experiments were also carried out where the concentrations of both CIF and colony-stimulating factor were varied. The results (Table 1) show that there is a direct relationship between the concentration of CIF required to produce a significant decrease in colony count (>20 per cent) and the concentration of stimulating factor present in the culture.

(b) *Incubation with CIF prior to agar culture*

Mouse bone marrow cells were incubated at a concentration of 4×10^6 cells per ml in collecting medium containing a concentration of CIF (10 per cent) known to inhibit colony formation if incorporated in the agar medium. Pre-treatment was carried out for 1 hour at 37° and 0°. The cells were then washed twice and cultured in the absence of CIF. Pre-treatment at either temperature had no effect on the colony-forming potential of the cells. Increasing the concentration of CIF in the pre-incubation medium (Table 2) failed to produce any inhibition of colony formation on subsequent culture.

(c) *Addition of CIF after initiation of cultures*

Table 3 shows the results of experiments where an inhibitory concentration of CIF was

TABLE 1

THE EFFECT OF ADDITION OF DIFFERENT CONCENTRATIONS OF CIF TO BONE MARROW CULTURES CONTAINING DIFFERENT AMOUNTS OF CSF ON THE NUMBER OF COLONIES PRESENT AFTER 7 DAYS INCUBATION (MEAN OF TWO EXPERIMENTS)

Concentration of CSF in culture	Colony count (per cent control) in cultures containing different volumes of CIF pool				
	0·4 ml	0·2 ml	0·1 ml	0·05 ml	0·02 ml
Neat	99	100	100	100	100
1/2	67*	100	94	99	100
1/4	59	65*	85	98	97
1/8	51	61	64*	100	96
1/16	52	52	62	59*	100
1/32	35	40	66	51	61*

* Indicates the concentration of CSF at which a given dose of CIF was able to produce a significant reduction (> 20 per cent) in colony number.

TABLE 2

COLONY FORMATION BY NORMAL MARROW CELLS PRETREATED FOR 1 HOUR AT 37° WITH DIFFERENT CONCENTRATIONS OF CIF

Concentration of CIF in pretreatment medium (per cent)	Colonies/10^5 cells
0	271 ± 18
10	271 ± 17
25	276 ± 12
50	279 ± 12

added to cultures at different times during the 7-day culture period. Addition of CIF to cultures up to 72 hours after initiation resulted in a significant decrease in the 7-day colony counts. Addition of CIF to cultures 96 hours or later after initiation had little effect on colony number. It is of interest that the same degree of inhibition was observed with the addition of CIF within the initial 72-hour period.

TABLE 3

EFFECT OF CIF ADDED AT DIFFERENT TIME INTERVALS AFTER CULTURE

Time of addition of CIF to cultures (hours)	Colonies/culture (average of 5 ± 1 SD)		
	Experiment 1	Experiment 2	Experiment 3
Nil	37 ± 5	110 ± 5	65 ± 7
0	16 ± 14	71 ± 5	25 ± 3
6	13 ± 2	70 ± 6	ND
24	18 ± 2	71 ± 4	28 ± 2
48	14 ± 2	75 ± 6	30 ± 4
72	16 ± 3	78 ± 3	34 ± 4
96	ND	96 ± 7	57 ± 6
120	ND	ND	57 ± 7

THE EFFECT OF CIF ON COLONY SIZE

Table 4 shows the result of an experiment where CIF was incorporated in the agar medium, and cultures removed after 3, 4 and 5 days incubation. Colony counts and cell counts on individual colonies were carried out from both inhibited and control cultures for each period of incubation. Normal cultures show considerable variation in colony size, making it difficult to establish significant differences. However, the results suggest that in addition to its effect on colony number CIF causes a decrease in the number of cells per colony which is greater as time progresses.

TABLE 4

COLONY NUMBER AND COLONY SIZE IN INHIBITED AND CONTROL CULTURE AFTER DIFFERENT PERIODS OF INCUBATION

Period of culture	Addition	Colonies/ culture	No. of cells/colony (range) Mean ± SD 30–40 sequential colonies	
3 Days	Saline	24 ± 2	9 ± 4	(4–15)
3 Days	CIF	12 ± 1	9 ± 4	(4–15)
4 Days	Saline	30 ± 3	16 ± 8	(8–38)
4 Days	CIF	16 ± 2	10 ± 5	(4–24)
5 Days	Saline	35 ± 5	22 ± 17	(5–80)
5 Days	CIF	24 ± 3	15 ± 10	(4–46)

COLONY CYTOLOGY

It is known that in the early stages of incubation (up to 3 days) colonies consist of granulocytic cells, and after 7 days incubation a large proportion of the colonies consist of macrophages. Individual colonies can be removed from cultures, stained and classified as either granulocyte, macrophage or mixed colonies of the two cell types (Metcalf, 1969). Colonies were removed from control cultures and cultures containing CIF after 3 and 7 days incubation for staining and microscopic examination. After 3 days incubation, both normal and inhibited colonies were made up entirely of cells of the granulocytic series, and no obvious differences in morphology were observed. However, after 7 days incubation, a large proportion of colonies from inhibited cultures were made up of unusual cells. These cells show the characteristic granular and vacuolated cytoplasm of the normal macrophage (Fig. 2a), but have a 'dumbell' or lobed nucleus (Fig. 2c) more typical of granulocytic cells. In addition some colonies contained a small number of multinucleated macrophages (Fig. 2b) but these were not a constant feature of CIF-containing cultures. Table 5 shows the results of investigations of the types of colony developing over a 5-day period of incubation in control cultures and cultures in which CIF was incorporated at the time of plating. This shows that in the presence of CIF colonies develop normal morphology during the early stages of culture, but thereafter show an increasing proportion of colonies made up of such unusual cells.

Experiments were also carried out where CIF was added to marrow cultures at various times during the 7-day incubation period. The results (Table 6) show that addition of CIF to cultures up to 5 days after plating resulted in the appearance of a significant proportion of colonies of unusual cytology, the greatest effect being observed when the addition was made between the 2nd and 4th day of culture. Although addition of CIF to

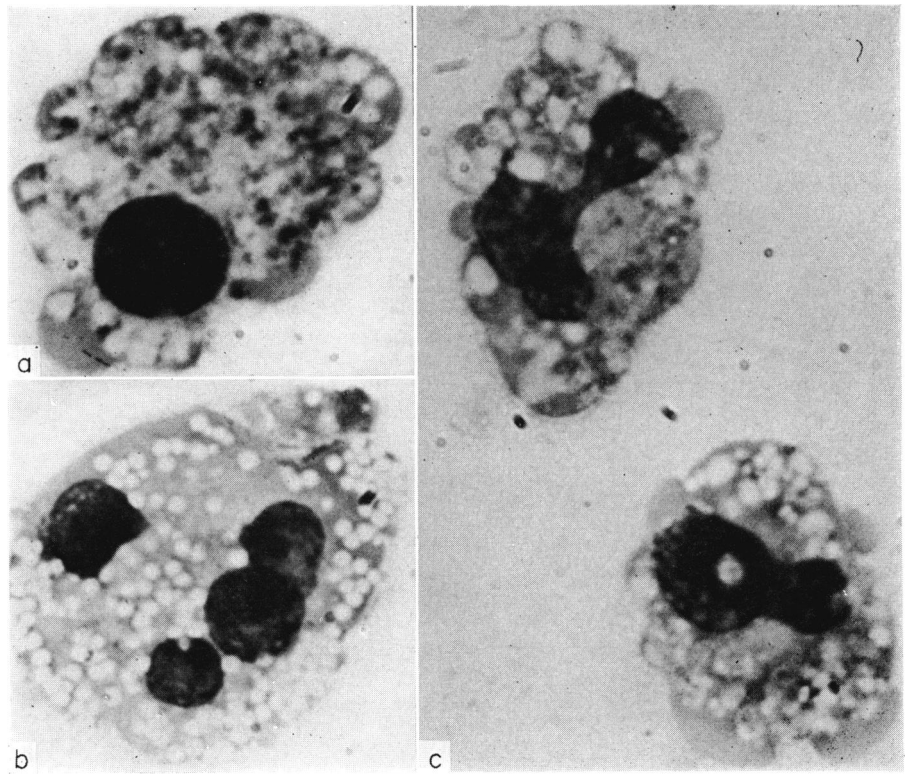

FIG. 2. (a) Typical colony macrophage from normal culture. May-Grunwald-Giemsa. (b) Multinucleate macrophage from CIF containing culture. May-Grunwald-Giemsa. (c) Unusual cells from 5-day CIF containing culture showing the coarse granular and vacuolated cytoplasm of macrophage type but lobed nucleus more typical of the granulocyte type. May-Grunwald-Giemsa. (×640.)

TABLE 5

COLONY CYTOLOGY IN CONTROL AND PARTIALLY INHIBITED CULTURES

Period of incubation	Culture	Colony type (per cent of total colonies)			
		Granulocyte	Mixed	Macrophage	Unusual
3 Days	Control	100	0	0	0
3 Days	Inhibited	100	0	0	0
4 Days	Control	53	40	7	0
4 Days	Inhibited	50	0	0	50
5 Days	Control	25	35	45	0
5 Days	Inhibited	0	0	0	100

cultures on the 4th and 5th days after plating had no significant effect on the 7-day colony count (Table 3), a large proportion of the colonies showed unusual cytology.

THE EFFECT OF CIF ON COLONY FORMATION BY L-CELLS

L-cells are a mouse-derived fibroblastic cell line which proliferate to form colonies when cultured in semi-solid agar media. Table 7 shows the results obtained when L-cells

were cultured in the presence of a concentration of CIF which inhibited bone marrow colony formation. Inclusion of CIF in such cultures had no effect on the formation of colonies by L-cells.

TABLE 6

THE EFFECT OF ADDITION OF CIF TO CULTURES AT DIFFERENT INTERVALS AFTER PLATING ON THE TYPE OF COLONY PRESENT AFTER 7 DAYS INCUBATION

Time of addition of CIF (hours)	Per cent colony type (from twenty colonies/group)			
	Granulocyte	Macrophage	Mixed	Unusual macrophage
Control	20	50	30	0
0	40	5	15	40
24	60	0	5	35
48	5	5	0	90
72	25	0	0	75
96	0	15	5	80
120	25	25	15	35

TABLE 7

THE EFFECT OF CIF ON THE FORMATION OF COLONIES BY L-CELLS IN SEMI-SOLID AGAR

Culture	Colonies/culture (average of 4 ± SD)	
	Marrow	L-cells
Control	64 ± 2	160 ± 9
CIF	29 ± 5	173 ± 8

DISCUSSION

The results reported in this paper show that the CIF obtained from mouse serum after inoculation with Poly I–Poly C inhibits the *in vitro* development of haemopoietic CFC in terms of colony number, size and differentiation. The relationship between CIF and interferon has been discussed elsewhere (McNeill and Killen, 1971; McNeill and Fleming, 1971; McNeill, Fleming and McCance, 1972) and it seems clear in view of the close similarity in physiological and physicochemical properties between the two that the effects on colony growth and development described here are mediated by interferon. This conclusion is supported by the presence of very large amounts of CIF in concentrated preparations of mouse interferon (McNeill and Gresser, unpublished observations). The present results suggest that CIF and CSF are directly antagonistic to each other in that the number of colonies which developed in the presence of both factors was dependent upon their relative concentrations (Table 1). In view of this relationship it would be interesting to know if CSF is antagonistic to the action of interferon in antiviral systems.

In order to exert its effect on colonies CIF, like CSF, must be present in the agar medium while the colonies are growing since no effect was demonstrable when bone marrow cells were pre-incubated with CIF in fluid medium prior to agar-culture (Table

2). This observation also indicates that the CIF preparation did not have a direct cytotoxic effect on colony-forming cells. Non-specific cytotoxity was also ruled out by the observation that a CIF concentration active against bone marrow colony growth was inactive when tested in colony cultures of the mouse-derived L-cells (Table 7).

The anti-cellular effect of interferon cannot however be regarded as specific for granulocyte-macrophage colony growth. Repeated administration of interferon has been reported to inhibit the growth of transplantable murine tumours *in vivo* (Gresser and Bourali, 1970; Gresser, Bourali, Chouroulinkov, Fontaine-Brouty-Boye and Thomas, 1970), and high concentrations have been shown to inhibit the growth of a variety of mouse cell lines *in vitro* (Gresser, Thomas and Brouty-Boye, 1971; Gresser, Thomas, Brouty-Boye and Macieira-Coelho, 1971; Lindahl-Magnusson, Leary and Gresser, 1971). Experiments, in collaboration with Dr Gresser, are in progress to determine the relative sensitivity of the different systems.

Cytological investigations on colonies developing in cultures of normal mouse bone marrow after varying periods of incubation (Metcalf, Bradley and Robinson, 1967; Metcalf, 1969) have shown that for the first 72 hours of incubation, colonies are composed predominantly of granulocyte-type cells. Thereafter, cells classifiable as macrophages appear, and after periods of 7 days incubation are the predominant type found in colonies. The relationship of macrophage colony cells to the earlier granulocytic type is not altogether clear. Since colonies arise from single cells either granulocytic-type cells can differentiate to macrophages or the two types of colony cell are derived from independent precursors in which case a mixed colony could develop by the incorporation of a macrophage precursor in a developing granulocytic colony. The former possibility is supported by evidence (Metcalf, 1971a) which indicates that single cells from 2-day-old granulocytic colonies when transferred to fresh cultures can give rise to colonies of either pure macrophages or mixed cell type. The present experiments indicate that CIF interferes with this process of differentiation. Colonies in cultures partially inhibited by CIF showed normal cytology after 3 days incubation (Table 6), although colony counts were reduced (Table 5). Cultures containing CIF incubated for periods in excess of 3 days failed to show the normal transition from granulocytic to macrophage colonies and developed an increasing proportion of colonies composed of unusual cells (Table 5, Fig. 2b and 2c). Treatment of cultures with CIF from 1–5 days after plating showed that the maximum number of unusual colonies was associated with CIF addition from the 2nd–4th day of the incubation, i.e. at the time when transition from granulocyte to macrophage type colony was taking place (Table 6). We use the term 'unusual' rather than 'abnormal' to describe these cells since they may be found in small numbers in normal 4–5-day-old colonies. It is possible, therefore, that if granulocyte precursors can differentiate into macrophages these cells may represent a transitional phase which in normal cultures is rapidly passed but which in CIF containing cultures is greatly prolonged. It is of interest that cultures stimulated by serum taken after inoculation of mice with bacterial endotoxin show a predominance of granulocyte-type colonies (Metcalf, 1971b), since in addition to causing a marked increase in CSF, endotoxin also induces an interferon response in the serum (McNeill and Killen, 1971).

Although interferon has been shown to inhibit the *in vitro* growth of a number of mouse cell lines, such inhibition does not effect the morphology of the cells (Gresser *et al.*, 1970). The experiments described above have not only demonstrated inhibition of the *in vitro* growth of haemopoietic CFC, but also have shown that CIF interferes with the normal

differentiation of these cells in culture. These results provide strong support for the suggestion that interferon may function as a haemopoietic regulator and that its antiviral function may be subordinate to this regulatory effect. The close similarity in physicochemical properties between colony-stimulating factor (Stanley and Metcalf, 1969), erythropoietin (Gordon, 1971), and interferon (Andrews, 1971) gives further support to this concept.

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